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(54) Title: IKK4

(57) Abstract: A novel inhibitory kappa B kinase (IKK) is disclosed herein as IKK4. Polynucleotides encoding IKK4, expression vectors comprising said polynucleotides and screening methods for identifying therapeutic modulators of IKK4 activity are also disclosed.

DESCRIPTION

IKK4

This invention relates to a novel IKK kinase protein, IKK4, nucleotides encoding for it, vectors and host cells containing the same and methods for screening for modulators of said IKK4 protein for treatment of conditions involving inflammation.

The transcription factor NF-kB controls the activation of various genes in response to pathogens and pro-inflammatory cytokines. Thus, for example, NF-kB is activated by various kinds of stimulation including tumour necrosis factor alfa (TNF alfa) and interleukin -1 (IL-1), bacterial LPS, viral infection, antigen receptor cross-linking of T and B cells, calcium ionophores, phorbol esters, UV radiation and free radicals (for reviews, see Varma et al., 1995, Genes Dev., 9, 2723-2735; Baueurerle and Baltimore, 1996, Cell, 87, 13-20), (see Figure 1). NF-kB in turn controls the activation of various genes in response to these stimuli. Activation of these various genes in turn may result in the production of cytokines, chemokines, leukocyte adhesion molecules, hematopoletic growth factors and may also effect development and cell death as well as cell survival (see Figure 1). Specifically, the transcription factor NF-kB controls the activation of various genes in response to pathogens and pro-inflammatory cytokines. The NF-kB activity is regulated through interaction with specific inhibitors, IkBs. Upon cell stimulation, the IkBs are rapidly phosphorylated and then undergo ubiquitin-mediated proteolysis, resulting in the release of active NF-kB (Baldwin, 1996, Annu. Rev. Immunol., 14, 649-681; Baueurerle and Baltimore, 1996, Cell, 87, 13-20), (see Figure 2). It has been reported that the 700 kDa complex specifically phosphorylated IkBα at S32 and S36 (Chen et al., 1996, Cell, 84, 853-862).

Several groups found that two kinases termed IKK1 (IKKα) and IKK2 (IKKβ), were the subunits of the kinase complex. These groups showed that the IKK immunoprecipitates, derived from the TNFα or IL-1 stimulated cells which are able to phosphorylate IkB in vitro. In addition to these observations, two groups reported that IKK1 and IKK2 purified from insect cells were able to phosphorylate IkB in vitro. These results suggested that IKK directly phosphorylates IkBs. The over expression of anti-sense IKK1, kinase-inactive

IKK1 or IKK2 resulted in the inhibition of NF-kB activation mediated by TNFα and IL-1. These results suggest that IKKs are critical kinases in the NF-kB activation pathway (May and Ghosh, 1998, Immunol. Today 19, 80-88; Stancovski and Baltimore, 1997, Cell, 91, 299-302). It has, however, not been understood how upstream signals are transmitted to the kinase complex, or whether different kinase complexes might exist to phosphorylate distinct IkBs.

NEMO (NF-kB essential modifier) and IKKγ (human homologue of the mouse NEMO) were isolated from purified IKK complex, and the inhibition of NEMO/IKKγ gene expression impaired the cytokine induced NF-kB activation via IKK1 and IKK2. In NEMO deficient cells, smaller complexes of Mr 3,000-4,000 are formed, though the normal complex is Mr 7,000-9,000, suggesting that NEMO/IKKγ physically link IkB kinase to upstream activators (Scheidereit, Nature, 1998, 395, 225-226).

The IKK-complex-associated protein (IKAP) was isolated from the IKK complexes. IKAP binds to IkB kinases and NIK and the complex, containing three kinases, leads to the maximum phosphorylation of IkB as compared to the complex containing one or two kinases. Accordingly, IKAP may act as scaffold proteins that link NIK or other molecules to IKK1 and IKK2 (Scheidereit, Nature, 1998, 395, 225-226). Accumulating evidence suggests that the IKK complex consists of several essential molecules, however, the molecular mechanisms that control the signalling complex were not well understood. Therefore, further association molecules were needed to complete the picture.

KIAA0151 was originally isolated from the KG-1 cDNA library (Nagase et al., 1995, DNA Res, 2, 167-174). KIAA0151 was identified as a potential Ser/Thr kinase, however, the importance of the molecule was not recognised. We have now found that KIAA0151 is similar to IKK1 and IKK2 using a computer homology analysis. KIAA0151, renamed IKK3, has a 21% homology with IKK1 and 23% with IKK2. IKK3 was able to phosphorylate IkB family proteins and directly phosphorylate IkB in vitro. The over expression of IKK3 leads to the activation of various inflammatory genes, such as IL-8, IL-6 and RANTES. These genes contain the NF-kB site in the gene regulation region. We know that IKK3 has an effect on IL-8 expression in Hela cells and also that IKK3 phosphorylates

NF-kB. Moreover, it is known that the NF-kB site has an important role in IL-8 regulation. Our results suggest a correlation between IKK3 and the NF-kB site of the IL-8 promoter that has previously been identified as an endogenous NF-kB binding site, further suggesting that IKK3 plays an important role in controlling the NF-kB site of the IL-8 promoter. These results lead to the conclusion that IKK3 is an important regulator of IL-8 gene regulation and thus activates genes that are important for the inflammatory diseases (see Table 1 below).

Table 1

Differences between IKK1, 2 and IKK3

	IKKε, β (also known as IKK1 & IKK2)	IKK3
Expression (mRNA)	Constitutive	Inducible by IL-1 and TNF-alfa
Source for in vitro phosphorylation	Mammalian and Insect cells	Mammalian and Bacterial cells
Spectrum	Unknown	IL-8, IL-6 and RANTES
Substrate Selectively	IkBα> IkBβ	IkBε IkBβ > IkBα
Enzymatic activity	Need for IL-1 or TNF alfa stimulation	No need for stimulation

Using a computer homology analysis we have now we identified a novel kinase, termed IKK4, that Is 49% similarity with IKK3, 25% identical to IKK1 and 24% identical to IKK2, at the amino acid level. The overexpression of IKK4 leads to the activation of the IL-8 reporter gene via NF-kB signalling pathway. We found that IKK4 is able to phosphorylate IκB family proteins using immunoprecipitation assays, and directly phosphorylate IκBβ in vitro using a GST-pull down assay. These results suggest that IKK4 activates the IL-8 gene as well as some other inflammatory related gene.

Accordingly this invention provides a novel isolated kinase protein, referred to herein as IKK4.

Nucleotide sequence of IKK4 reveals a 2187 bp open reading frame which encodes a 729 amino acid protein (see SEQ.I.D.NO:1). At the amino acid level, IKK4 is 49 % identical to IKK3, and 25% identical to IKK1 and 24% identical to IKK2. On the other hand, IKK1 has a 52% identity to IKK2. The amino acid sequence of IKK4 revealed that it has a potential kinase domain, though IKK3 has two leucine zippers, and IKK1/IKK2 have one leucine zipper as reported previously. IKK1 and IKK2 have an HLH domain at the C-terminal region, however, we are unable to find a typical HLH domain, which were found in IKK1 and IKK2 (Figs. 3 and 4). The kinase domain of IKK3 and IKK4 have 73% identity at the amino acid level. In addition, two domains in IKK3 and IKK4 were found similarity in the amino acid level. We termed these regions as HR1 and HR2. The IKK4 HR1 region (amino acids 300-450) and IKK3 HR1 region (amino acids, 300-450) are 43% identical at the amino acid level. The IKK4 HR2 region (amino acids, 562-609) and IKK3 HR2 region (amino acids, 552-600) are 50% identical at the amino acid level.

One aspect of the invention therefore provides an isolated IKK4 kinase protein or a variant thereof. The amino acid sequence of this isolated IKK4 kinase protein is shown in SEQ I.D.No: 1.

By the term " isolated" we mean that the protein herein exists in a physical milieu distinct from that in which it occurs in nature. For example, the protein maybe substantially isolated with respect to the complex cellular milieu with which it is normally associated with. The absolute level of purity is not critical and maybe readily determined by the skilled person according to the use to which the protein is put.

Included within the invention are variants of the IKK4 kinase protein. Such variants include fragments, analogues, derivatives and splice variants. The term "variant" refers to a protein or part of a protein which retains substantially the same biological function or activity as IKK4.

Fragments can include a part of IKK4 which retains sufficient identity of the original protein to be effective for example in a screen. Such fragments may be probes such as the ones described hereinafter for the identification of the full length protein. Fragments may be fused to other amino acids or proteins or may be comprised within a larger protein. Such a fragment may be comprised within

a precursor protein designed for expression in a host. Therefore, in one aspect the term fragment means a portion or portions of a fusion protein or polypeptide derived from IKK4.

Fragments also include portions of IKK4 characterised by structural or functional attributes of the protein. These may have similar or improved chemical or biological activity or reduced side-effect activity. For example, fragments may comprise an alpha, alpha-helix or alpha-helix-forming region, beta sheet and beta-sheet-forming region, turn and turn-forming regions, coil and coil-forming regions, hydrophilic regions, hydrophobic regions, amphipathic regions (alpha or beta), flexible regions, surface-forming regions, substrate binding regions and regions of high antigenic index.

Fragments or portions may be used for producing the corresponding full length protein by peptide synthesis.

Derivatives include naturally occurring allelic variants. An allelic variant is an alternate form of a protein sequence which may have a substitution, deletion or insertion of one or more amino acids, which does not substantially alter the function of the protein. Derivatives can also be non-naturally occurring proteins or fragments in which a number of amino acids have been substituted, deleted, added, rearranged or modified. Proteins or fragments which have at least 70% identity to IKK4 are encompassed within the invention. Preferably, the identity is at least 80%, more preferably at least 90% and still more preferably at least or greater than 95% identity for example 97%, 98% or even 99% identity to IKK4.

Analogues include but are not limited to precusor proteins which can be activated by cleavage of the precursor portion to produce an active mature protein or a fusion with a compound such as polyethylene glycol or a leader/secretary to aid purification.

A splice variant is a protein product of the same gene, generated by alternative splicing of mRNA, that contains additions or deletions within the coding region (Lewin N (1995) Genes V Oxford University Press, Oxford, England). The

present invention covers splice variants of the IKK4 kinase protein that occur naturally and which may play a role in the control of inflammation.

The protein or variant of the present invention may be a recombinant protein, a natural protein or a synthetic protein, preferably a recombinant protein.

A further aspect of the invention provides an isolated (as defined *supra*) nucleotide sequence which encodes a mammalian IKK4 protein as described above, or a variant thereof. Also included within the invention are anti-sense nucleotides or complementary strands.

Preferably, the nucleotide sequence encodes the rat, murine or human IKK4 protein. The nucleotide sequence preferably comprises the sequence of the coding portion of the nucleotide sequence shown in SEQ I.D N0: 2.

A nucleotide sequence encoding an IKK4 protein of the present invention may be obtained from a cDNA or a genomic library derived from the human fetus Marathon-Ready cDNA (Clonetech).

The nucleotide sequence may be isolated from a mammalian cell (preferably a human cell), by screening with a probe derived from the rat,murine or human IKK4 sequence, or by other methodologies known in the art such as preliminary chain reaction (PCR) for example on genomic DNA with appropriate oligonucleotide primers derived from or designed based on the rat, murine or human IKK4 sequence and/or relatively conserved regions of known IKK3 proteins. A bacterial artificial chromosome library can be generated using rat or human DNA for the purposes of screening.

The nucleotide sequence of the present invention may be in form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single-stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the IKK4 protein or variant thereof may be identical to the coding sequence set forth in SEQ.I.D. NO:2, or maybe a different coding

sequence which as a result of the redundancy or degeneracy of the genetic code, encodes the same protein as the sequences set forth therein.

A nucleotide sequence which encodes an IKK protein may include:

a coding sequence for the full length protein or any variant thereof, and additional coding sequence such as a leader or secretory sequence or a pro-protein sequence: a coding sequence for the full length protein or any variant thereof (and optionally additional coding sequence) and non-coding sequences, such as intrans or non-coding sequences 5' and/or 3' of the coding sequence for the full length protein. The invention also provides nucleotide variants, analogues, derivatives and fragments which encode IKK4. Nucleotides are included which preferably have at least 70% identity over the entire length to IKK4. More preferred are those sequences which have at least 80% identity over their entire length to IKK4. Even more preferred are polynucleotides which demonstrate at least 90% for example 95%, 97%, 98% or 99% identity over their entire length to IKK4

The present invention also relates to nucleotide probes constructed from the nucleotide sequence of an IKK protein or variant thereof. Such probes could be utilised to screen a cDNA or genomic library to isolate a nucleotide sequence encoding an IKK4 protein. The nucleotide probes can include portions of the nucleotide sequence of the IKK4 protein or variant thereof useful for hybridising with mRNA or DNA in assays to detect expression of the IKK4 protein or localised its presence on a chromosome using for example flourescence in situ hybridisation (FISH).

The nucleotide sequences of the invention may also have the coding sequence fused in frame to a marker sequence which allows for purification of the protein of the present invention such as hexa-histadine tag or hemagglutinin (HA) tag, Myc-tag, T7-tag, double MYC-tag, double HA-tag and double T7-tag expression vectors or allows determination in screening assays of effective blockage of IKK4 or it's modulation.

Nucleotide molecules which hybridise to IKK4 or to complementary nucleotides thereto also form part of the invention. Hybridisation is preferably under stringent hybridisation conditions. One example of stringent hybridisation conditions which is sometimes used is where attempted hybridisation is carried out at a temperature of from about 35°C to about 65°C using a salt solution which is about 0.9 mol. However, the skilled person will be able to vary such conditions as appropriate in order to take into account variables such as probe length, base composition, type of ions present etc. The nucleotide sequence of the present invention may be employed for producing the IKK4 protein or variant thereof by recombinant techniques. Thus, for example the nucleotide sequence may be included in any one of a variety of expression vehicles or cloning vehicles, in particular vectors or plasmids for expressing a protein, such vectors include chromosomal, non-chromosomal and synthetic DNA sequences. Examples of suitable vectors include derivatives of bacterial plasmids: phage DNA: yeast plasmids; vectors derived from combinations of plasmids and phage DNA and viral DNA. However, any other plasmid or vector may be used as long as it is replicable and viable in the host.

More particularly, the present invention also provides recombinant constructs comprising one or more of the nucleotide sequences as described above. The constructs comprise an expression vector, such as a plasmid or viral vector into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment the construct further comprises one or more regulatory sequences to direct messenger mRNA synthesis, including, for example a promoter operably linked to the sequence. Suitable promoters include: CMV, LTR, actin or SV40 promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector may contain an enhancer and a ribosome binding site for translation initiation and transcription terminator.

Large numbers of suitable vectors and promoters/enhancers, will be known to those of skill in the art, but any plasmid or vector, promoter/enhancer may be used as long as it is replicable and functional in the host.

Appropriate cloning and expression vectors for use with prokaryotic and eurkaryotic hosts include mammalian expression vectors, insect expression vectors, yeast expression vectors, bacterial expression vectors and viral expression vectors and are described in Sambrook *et al*, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor, NY, (1989). The vector may also include appropriate sequences for selection and/or amplification of expression. For this the vector will comprise one or more phenotypic selectable/amplifiable markers, such markers are also well known to those skilled in the art.

In a further embodiment, the present invention provides host cells capable of expressing a nucleotide sequence of the invention, the host cell can be, for example, a higher eukaryotic cell, such as mammalian cell or a lower eukaryotic cell, such as a yeast cell or a prokaryotic cell such as a bacterial cell. Suitable prokaryotic hosts for transformation include *E-coli*. Other examples include viral expression vectors, insect expression systems and yeast expression systems.

Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention.

The IKK4 protein is recovered and purified from recombinant cell cultures by methods known in the art, including ammonium sulfate or ethanol precipitation, acid extraction, and ion or cation exchange chromotography, phosphocellulose chromotography and lecitin chromotography. Protein refolding steps may be used, as necessary, in completing configuration of the mature protein. Finally high performance liquid chromotography (HPLC) can be employed for final purification steps.

The present invention also provides antibodies specific for the IKK4 protein. The term antibody as used herein includes all immunoglobulins and fragments thereof which contain recognition sites for antigenic determinants of proteins of the present invention. The antibodies of the present invention may be polyclonal or preferably monoclonal, may be intact antibody molecules or fragments containing the active binding region of the antibody, e.g. Fab or $(Fab)_2$. The present invention also includes chimaeric, single chain and

humanised antibodies and fusions with non-immunoglobulin molecules. Various procedures known in the art may be used for the production of such antibodies and fragments.

The proteins, their variants especially fragments, derivatives, or analogues thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. Antibodies generated against the IKK4 protein can be obtained by direct injection of the polypeptide into an animal, preferably a non-human. The antibody so obtained will then bind the protein itself. In this manner, even a sequence encoding only a fragment of the protein can then be used to generate antibodies binding the whole native protein. Such antibodies can be used to locate the protein in tissue expressing that protein.

The antibodies of the present invention may also be of interest in purifying an IKK4 protein and accordingly there is provided a method of purifying an IKK4 protein or any portion thereof which method comprises the use of an antibody of the present invention.

The present invention also provides methods of identifying modulators of the IKK4 protein. Screens can be established for IKK4 enabling large numbers of compounds to be studied. High throughput screens may be based on $_{14}C$ guanidine flux assays and flourescence based assays as described in more detail below. Secondary screens may involve electrophysiological assays utilising patch clamp technology or two electrode voltage clamps to identify small molecules, antibodies, peptides, proteins or other types of compounds that inhibit, block, or otherwise interact with the IKK4 protein. Tertiary screens may involve the study of the modulators in well characterised rat and mouse models of inflammation. These models of inflammation include, but are not restricted to inflammatory models (murine) atopic dermatitis models (murine and rat), repeated-induced type dermatitis model (murine) and allergic asthma models (murine and guinea pig). For example, screens may be set up based on an in vitro phosphorylation system using bacterially expressed IKK4 proteins (see Example 3 and Figure 12). This system may be used to screen for modulators of the IKK4 kinase activity and then subsequently testing the effect of potential modulators of IKK4 on gene expression, specifically the expression of IL-8 using

cell based assay systems. Finally the efficacy of these modulators in relation to inflammatory or allergic diseases may be tested on models of inflammation.

The invention therefore provides a method of assaying for a modulator comprising contacting a test compound with the IKK4 protein and detecting the activity or inactivity of the IKK4 protein. Preferably, the methods of identifying modulators or screening assays employed transformed host cells that express the IKK4 protein. Typically, such assays will detect changes in the activity of the IKK4 protein to the test compound. thus identifying modulators of the IKK4 protein.

Modulators of the present invention maybe an agonist, antagonist or mimetic of IKK4 activity.

In general, a test compound is added to the assay and its effect on IKK4 is determined or the test compound's ability to competitively bind to the IKK4 is assessed. Test compounds having the desired effect on the IKK4 protein are then selected.

IL-8 is involved in diseases involving inflammation and allergies. Specifically, asthma, atopic dermatitis, arthritis, rheumatoid arthritis, systemic lupus erythematosus, LPS - induced contact dermatitis, glomerulonephritis, gout and other inflammation-related diseases.

The invention therefore provides a modulator of a protein or a variant thereof as described above identifiable by a method described above for use in therapy. The invention further provides use of a modulator of an IKK4 protein optionally identifiable by a method described above for the manufacture of an anti-inflammatory medicament. Moreover the invention provides a method of treatment which comprises administering to a patient an effective amount of a modulator of a protein as described above. More specifically, the invention provides a method of treating diseases related to inflammation, such as asthma, atopic dermatitis, arthritis, rheumatoid arthritis, systemic lupus erythematosus, LPS - induced contact dermatitis, glomerulonephritis and gout.

Complementary or anti-sense strands of the nucleotide sequences as herein above defined can be used in gene therapy. For example, the cDNA sequence of fragments thereof could be used in gene therapy strategies to down regulate the IKK4 protein. Anti-sense technology can be used to control gene expression through triple-helix formation of anti-sense DNA or RNA, both of which methods are based on binding of a nucleotide sequence to DNA or RNA.

A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription thereby preventing transcription and the product of the sodium channel. The anti-sense RNA oligonucleotide hybridises to the messenger RNA *in vivo* and blocks translation of the messenger RNA into the IKK4 protein.

The regulatory regions controlling expression of the IKK4 protein could be used in gene therapy to control expression of a therapeutic construct in cells expressing the IKK4 protein.

Figures

Brief Description of the Figures:

Figure 1

Outside factors stimulating expression of NF-kB as well as the effect of NF-kB on various biological events.

Figure 2

Regulation of NF-kB activity.

Figure 3

Schematic representation of IKK alpha, beta, IKK3 and IKK4 (KD = kinase domain; LZ = leucine zipper, HLH = helix-loop-helix). IKK3 is 25% identical to IKK1 and 24% identical to IKK2 at the amino acid level. IKKα has a 52% identity to IKKβ at the amino acid level.

Figure 4

Northern blot analysis:

The human tissue filter for the northern blot (gene hunter, TOYOBO) was probed with the IKK4 specific primers.

Figure 5

In vitro phosphorylation of IkB proteins by IKK4.

Figure 6

IKK4 directly phosphorates TRIP9.

The bacterially expressed GST-IKK4 were incubated with the bacterially expressed GST, GST-TRIP9, -TRIP9/AA and [γ^{-32}P] ATP for 30 min at 30°C. Proteins were separated by SDS-PAGE, stained with Coomassie blue and analyzed by autoradiography

Figure 7

IKK4 controls an essential step in NF-kB signalling pathway.

Example 1 Materials and methods

Cells and transfection

Hela cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum. DNA transfection into cells was performed via DOSPER transfection according to the manufacture's instructions.

Vector construction

Two EST cDNA fragments (AA361478 and AA173512), similar to IKK3 were derived from one cDNA. The full lenght IKK4 cDNA was obtained by PCR from the human Jurkat cell line using Marathon cDNA Amplification kit (Clonetech). The 5' half fragment (5'primer AP1 and 3' primer G198) and 3' half fragment (5' primer G197 and AP1) were amplified by PCR and digested with Nhe I. The resulting fragment was subcloned into pCR2.1-TOPO vector. To attach a Not I site at the both ends of the cDNA, the cDNA was amplified by PCR with 5' primer G205 and 3' primer G213. The cDNA was sbcloned into a pCR2.1-TOPO vector.

G197: 5'-ACGAAGGGCGACGCTTAGTCTTAGAACC-3'

G198: 5'-GTGCGTCATAGCTTTTGTGGCATGGT-3'

G205: 5'-CCCCCCGCGGCCGCCACCATGCAGAGCACTTCTAATCATCTG-3'

G213: 5'-CCCCCCGCGGCCGCCCTAAAGACAGTCAACGTTGCGAAGGCC-3'

The cDNA fragment was digested with *Not*I and the fragment was subcloned into pGEX-4T (Pharmacia).

All PCR-derived sequences used in this study were confirmed by the Sanger method.

Example 2

Northern blot analysis

Expression of IKK4

To test the expression of IKK4 in human tissues, we performed Nothern blot analysis. The IKK4 expression was detected in the Brain, Liver, Pancreas, Placenta and Lung, but not in the Heart (Fig. 6).

Example 3

In vitro phosphorylation

Hela cells were transiently expressed with the double T7-tagged IKK4 (DT7-IKK4) expression vector. Thirty-six hours after transfection, cells were prepared by lysis with TNE buffer (10 mM Tris-HCl, pH 7.8; 1% NP-40, 0.15 M NaCl; 1 mM EDTA; 10 mM NaF, 2mM Na3VO4, 10 mM PNPP and complete) and the IKK4 protein was immunoprecipitated with anti-T7 antibody. Purified DT7-IKK4 was used for in vitro kinase reactions with bacterially expressed GST, GST-IκBα (1-54), -IκBβ (1-44), -IκBε (140-244), -TRIP9 (1-44) and [γ-^{32}P] ATP. The alanine-substitution mutants GST-IκBα (IκBα/AA), -IκBβ (IκBβ/AA), -TRIP9 (1-44, AA), -IκBε (IκBε/AA1 and IκBε/AA2) were used as control proteins. Proteins were separated by SDS-PAGE, stained with Coomassie blue and analyzed by autoradiography.

In the case of direct phosphorylation of IkB by IKK4, the bacterially expressed GST-DT7-IKK4 was used as a kinase. 250 ng of purified kinase solution was used for in vitro kinase reactions with a 500 ng of bacterially expressed GST, GST-TRIP9 (1-44), -TRIP (1-44, AA) and [γ-^{32}P] ATP. Proteins were separated by SDS-PAGE, stained with Coomassie blue and analyzed by autoradiography.

Results:

To test whether IKK4 is able to phosphorylate IκBs, we transfected Hela cells with the double T7-tagged IKK4 expression vector. Thirty-six hours after transfection, IKK4 proteins were immunoprecipitated with anti-T7 antibody. Purified IKK4 was used for in vitro kinase reactions, containing [γ-^{32}P] ATP and bacterially expressed GST, GST-IκBα (1-54 a.a.), -IκBβ (1-44 a.a.), -IκBε (140-244 a.a.), and -TRIP9 (1-44 a.a.).

The immunoprecipitates of the double T7-tagged IKK4 preferentially phosphorylate GST-IκBβ rather than GST-IκBα and IκBε, while the immunoprecipitates did not phosphorylate GST in vitro (Fig. 7). Previously, it has been reported that residues Ser 32/Ser36 in IκBα and Ser19/Ser23 in IκBβ become phosphorylated upon TNF-α or IL-α/β stimulation (Brockman et al., 1995; Brown et al., 1995; Traenchner et al., 1995; Whiteside et al., 1995; DiDonato et al., 1996). To test whether these residues are phosphorylated by

IKK4, we mutated the residues in IκBα to alanine. The corresponding residues of IκBβ and IκBε were also mutated to alanine. The alanine-substitution mutants GST-IκBα (1-54, AA), -IκBβ (1-44, AA), were not phosphorylated at all (Fig. 7, lanes 3, 5). On the other hand, the alanine mutants of GST-IκBε (140-244, AA1 and AA2) were partially phosphorylated (Fig. 7, lanes 7 and 8).

These data suggest that IKK4 is involved in the IκB phosphoryaltion. However, it is still unclear whether IKK4 directly phospholylates IκBs or requires some other molecules to modify the IκBs. We next examined whether IKK4 directly phosphorylates IκBs using a GST-pull down assay. We constructed GST-DT7-IKK4 and expressed the GST-IKK4 protein in *E. coli*. The affinity purified GST-IKK4 was used for the in vitro phosphorylation assay. The bacterially expressed GST, GST-TRIP9 or GST-TRIP9/AA were incubated with GST-IKK4 and [γ-^{32}P] ATP. The proteins were separated by SDS-PAGE, stained with Coomassie blue and the gel was analyzed by autoradiography. Figure 8 shows that GST-IKK4 phosphorylates GST-TRIP9 but not GST and GST- TRIP9/AA. These results indicate that IKK4 directly phosphorylate serine 19 or 23 of IκBβ.

IKK4 regulates the NF-κB site of IL-8

Previously, we reported that IKK3 is able to control the IL-8 reporter gene via the NF-kB binding site. To test whether IKK4 regulates the NF-κB site of IL-8, DT7-IKK4 was transiently expressed in the human 293T cells with the IL-8 reporter gene. Transient expression of IKK4 activates the IL-8 reporter gene, while IKK4 is unable to activate the mutated reporter gene that contains a mutation at the NF-κB site of the IL-8 promoter (Fig. 9). These observations indicate that IKK4 is one of a critical kinases for the IL-8 gene regulation via the NF-κB site.

C L A I M S

1. The protein having the amino acid sequence in Seq. I.D No. 1, or a variant thereof.

2. An IKK4 kinase protein or variant thereof according to claim 1 for use in a method for screening for agents with anti-inflammatory activity.

3. A nucleotide sequence encoding the protein of claim 1 or a variant thereof, or a nucleotide sequence which is complementary thereto.

4. A nucleotide sequence encoding the protein of claim 1 as shown in Seq. I.D No. 2, or a variant thereof, or a nucleotide sequence which is complementary thereto.

5. The nucleotide sequence of either claim 3 or 4, which is a cDNA sequence.

6. A nucleotide sequence that hybridises to any part of a nucleotide strand referred to in either of claims 3 to 5.

7. An expression vector comprising a nucleotide sequence according to any one of claims 3 to 6, which is capable of expressing a IKK4 kinase protein or a variant thereof.

8. A stable cell line comprising a vector according to claim 7.

9. A cell line according to claim 8 which is a Hela cell line.

10. An antibody specific for a protein as claimed in claim 1.

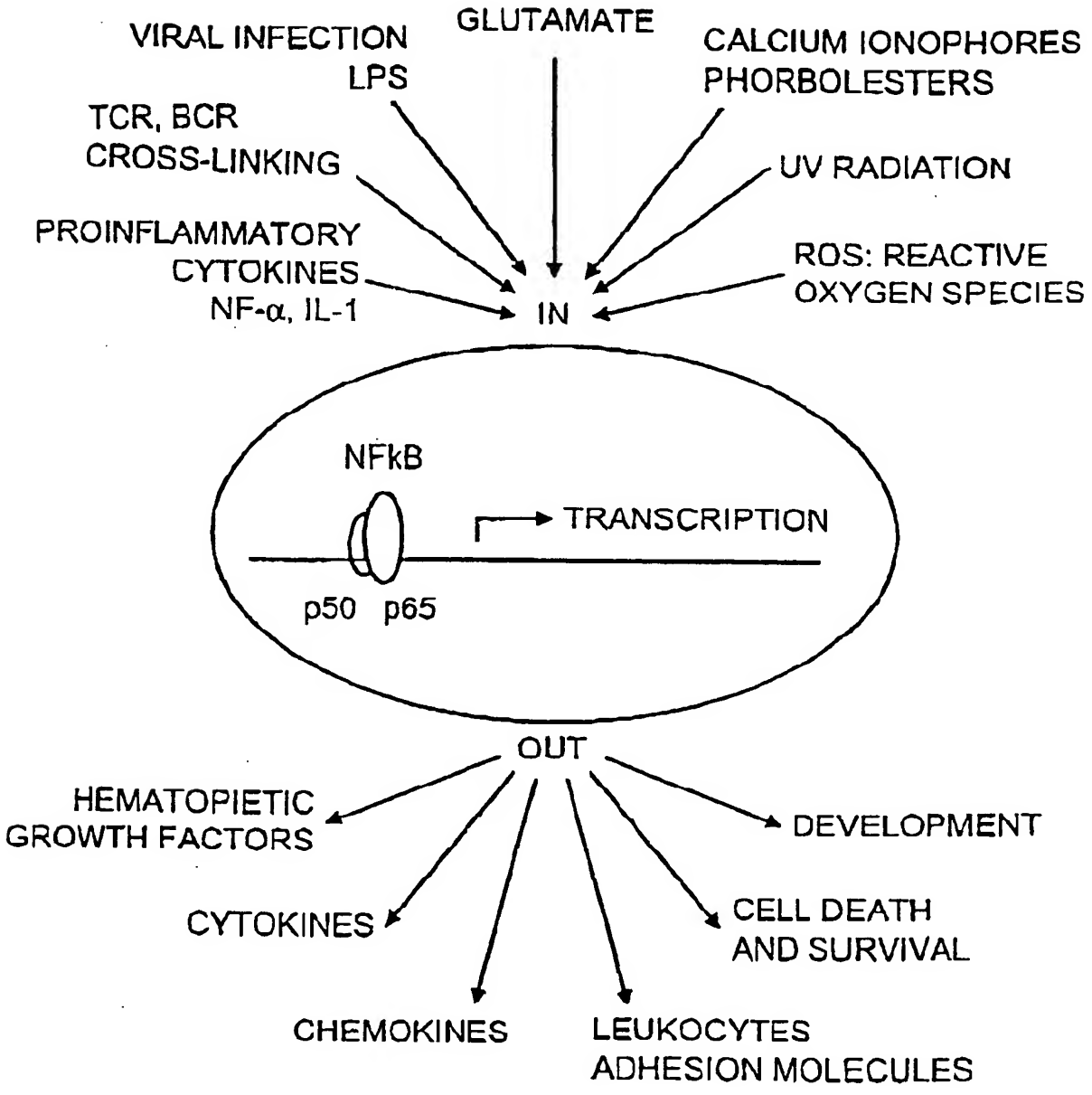
11. A method for identification of a compound which exhibits IKK4 kinase modulating activity, comprising contacting a IKK4 kinase protein according to claims 1 or 2 with a test compound and detecting modulating activity or inactivity.

12. A compound which modulates the protein of claim 1, identifiable by a method according to claim 11.

13. A method of treatment or prophylaxis of a disorder which is responsive to modulation of IKK4 kinase activity in a mammal, which comprises administering to said mammal an effective amount of a compound identifiable by the method according to claim 11.

14. Use of a compound identifiable by the method according to claim 11 in a method of formulating a medicament for treatment or prophylaxis of a disorder which is responsive to the modulation of IKK4 kinase activity in a mammal.

15. A method of producing an IKK4 kinase protein comprising introducing into an appropriate cell line a suitable vector or vectors comprising a nucleotide sequence encoding for IKK4 or variants thereof, under conditions suitable for obtaining expression of the protein or variants.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CALCIUM IONOPHORES
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TCR, BCR
CROSS-LINKING
UV RADIATION
PROINFLAMMATORY
CYTOKINES
NF-α, IL-1
IN
ROS: REACTIVE
OXYGEN SPECIES
NFkB
TRANSCRIPTION
p50 p65
OUT
HEMATOPIETIC
GROWTH FACTORS
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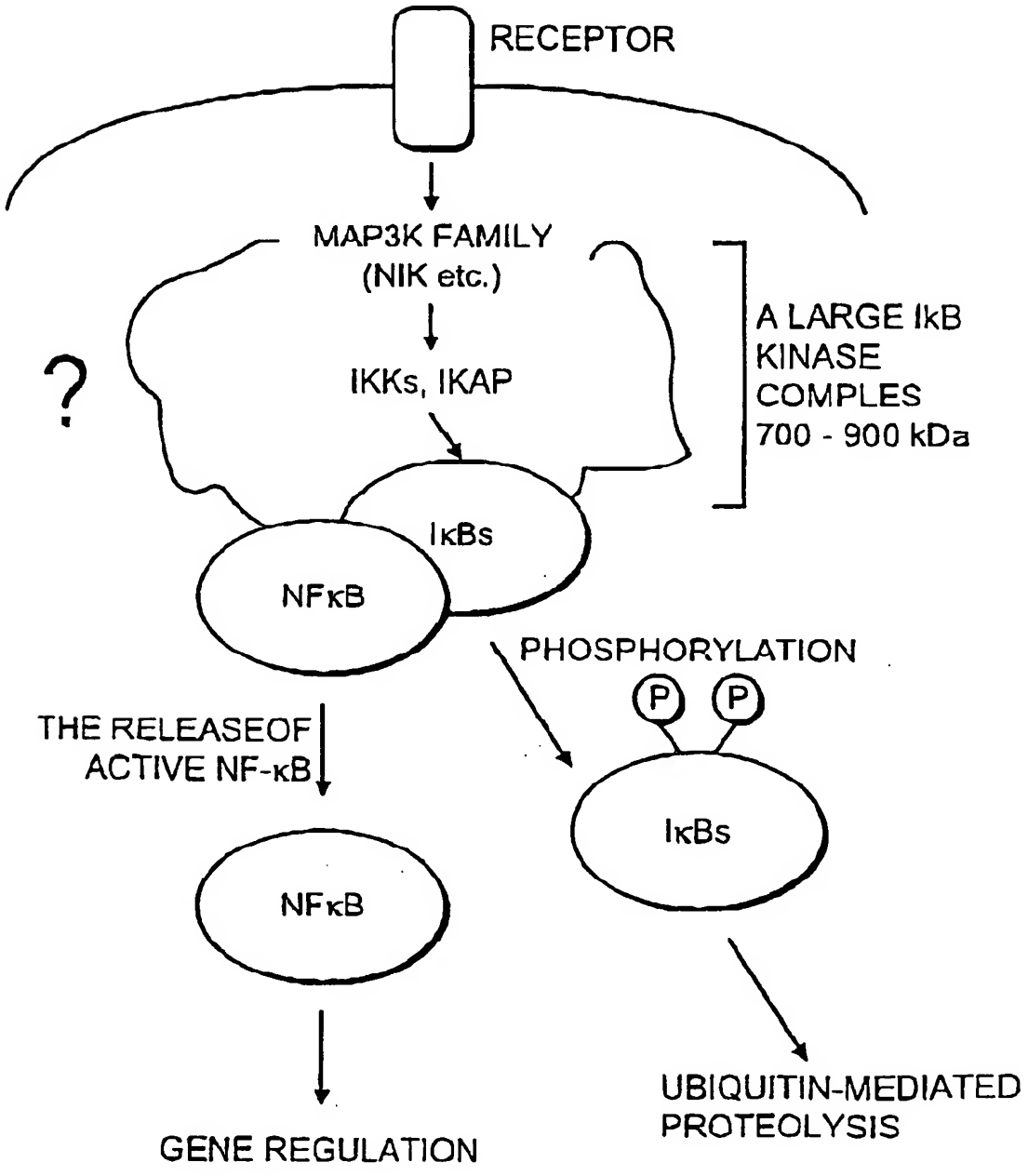
AND SURVIVAL
CHEMOKINES
LEUKOCYTES
ADHESION MOLECULES

FIG. 1


RECEPTOR
MAP3K FAMILY
(NIK etc.)
IKKs, IKAP
?
A LARGE IkB
KINASE
COMPLES
700 - 900 kDa
IκBs
NFκB
PHOSPHORYLATION
P
P
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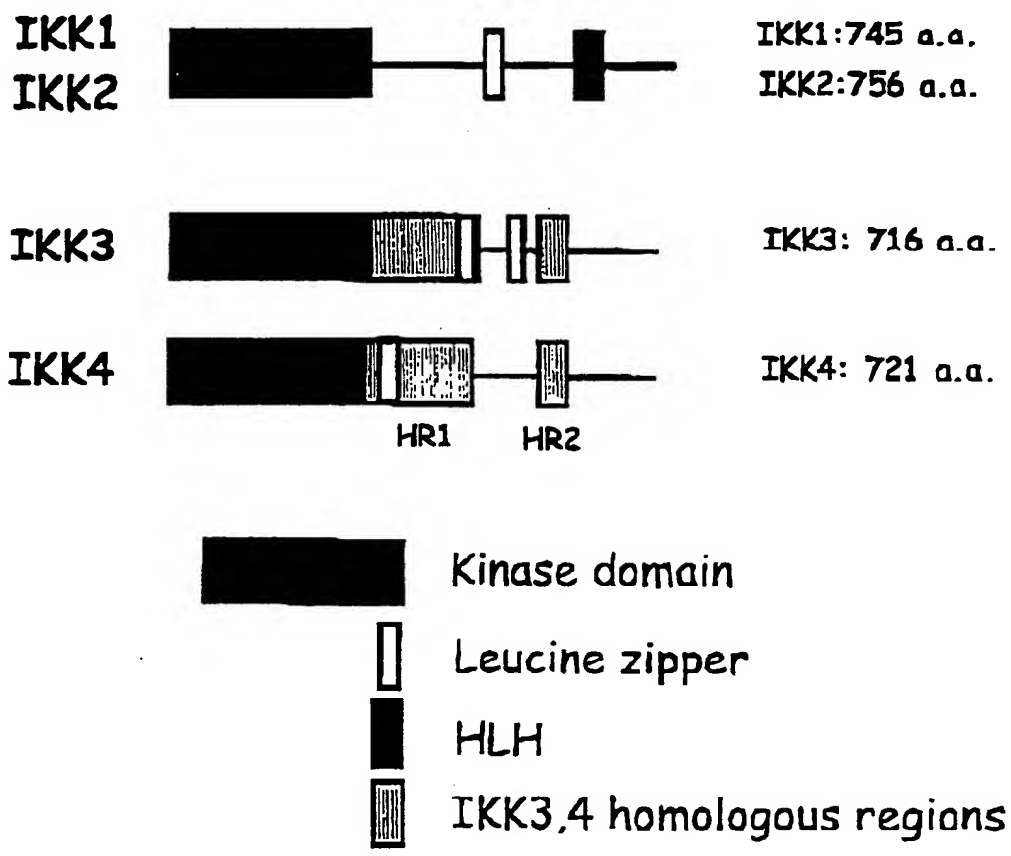
GENE REGULATION
UBIQUITIN-MEDIATED
PROTEOLYSIS

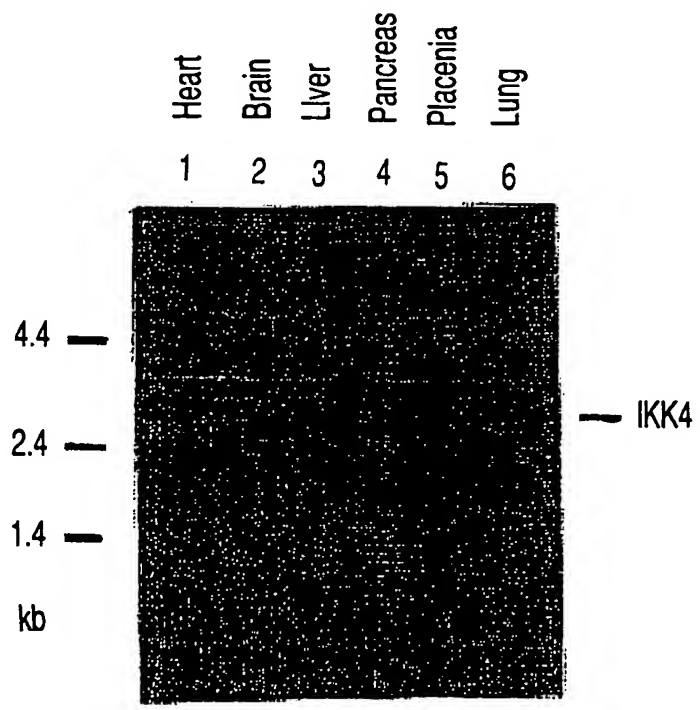
FIG. 2



Schematic representation of IKK1, IKK2, IKK3 and IKK4.

KD, kinase domain; LZ, leucine zipper, HLH, helix-loop-helix. IKK4 is 21% identical to IKK1 and 23% identical to IKK2 at the amino acid level. IKK1 has a 52% identity to IKK2 at the amino acid level.

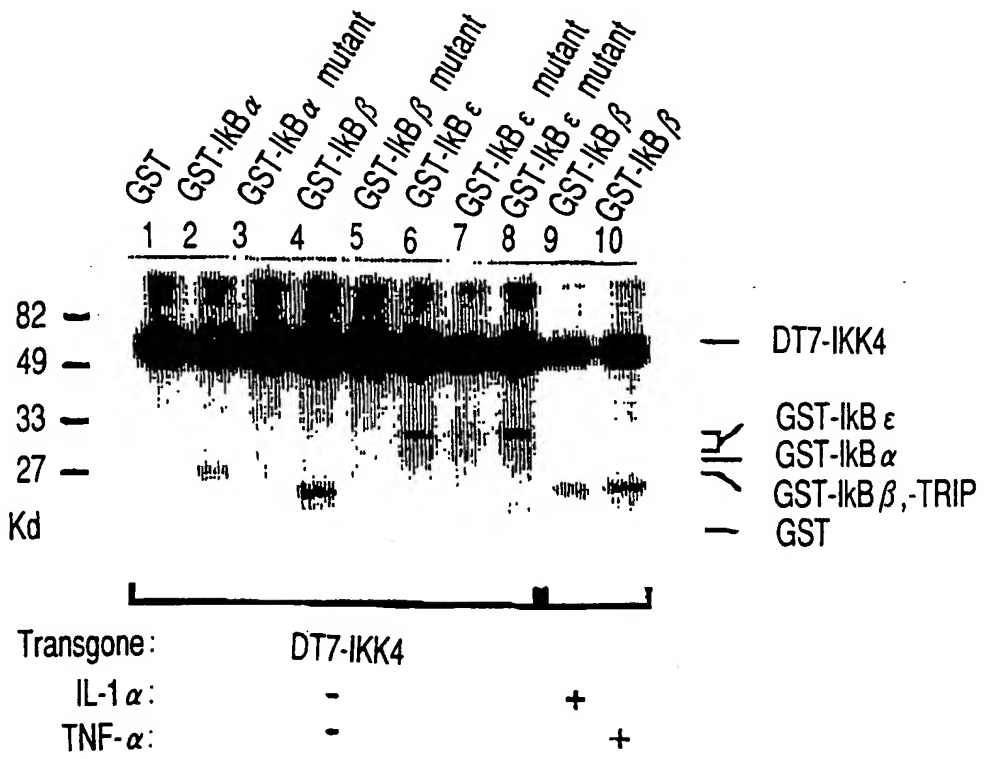
FIG. 3



Northern blot analysis.

The human tissue filter for northern blot (gene hunter, TOYOBO) was probed with the IKK4 specific primers.

FIG. 4



In vitro phosphorylation of IκB proteins by IKK4.

Hela cells were transiently expressed with the double T7-tagged IKK4 expression vector. Thirty-six hours after transfection, IKK4 proteins were immunoprecipitated with anti-T7 antibody. Purified DT-IKK4 was used for in vitro kinase reactions with bacterially expressed GST, GST-IκBα. -IκBαAA, -IκBβ, -TRIP9/AA, -IκBε, -IκBε/AA1 and -IκBε/AA2 and [γ-^{32}P] ATP. Proteins were separated by SDS-PAGE, stained with Coomassie blue and analyzed by autoradiography.

FIG. 5

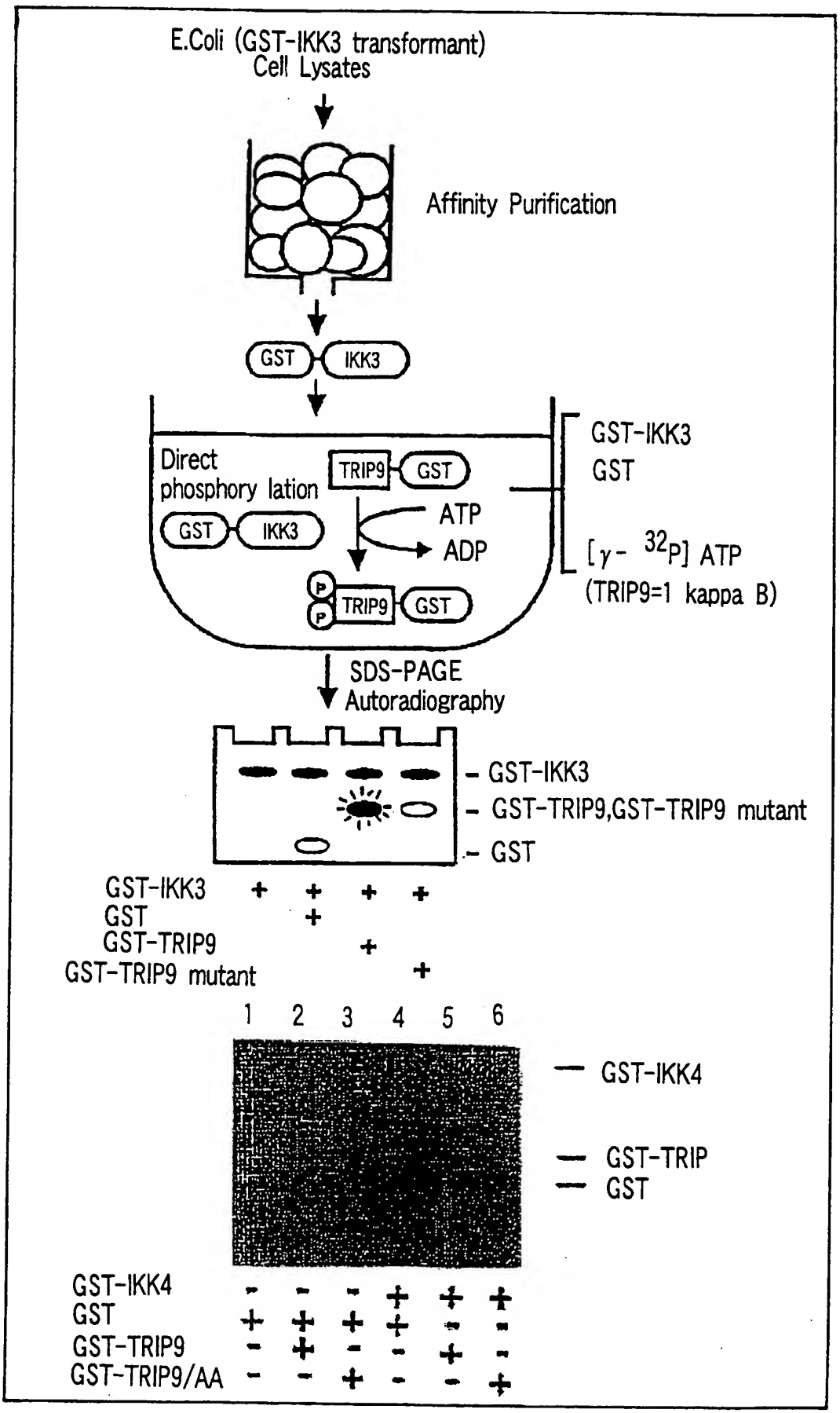
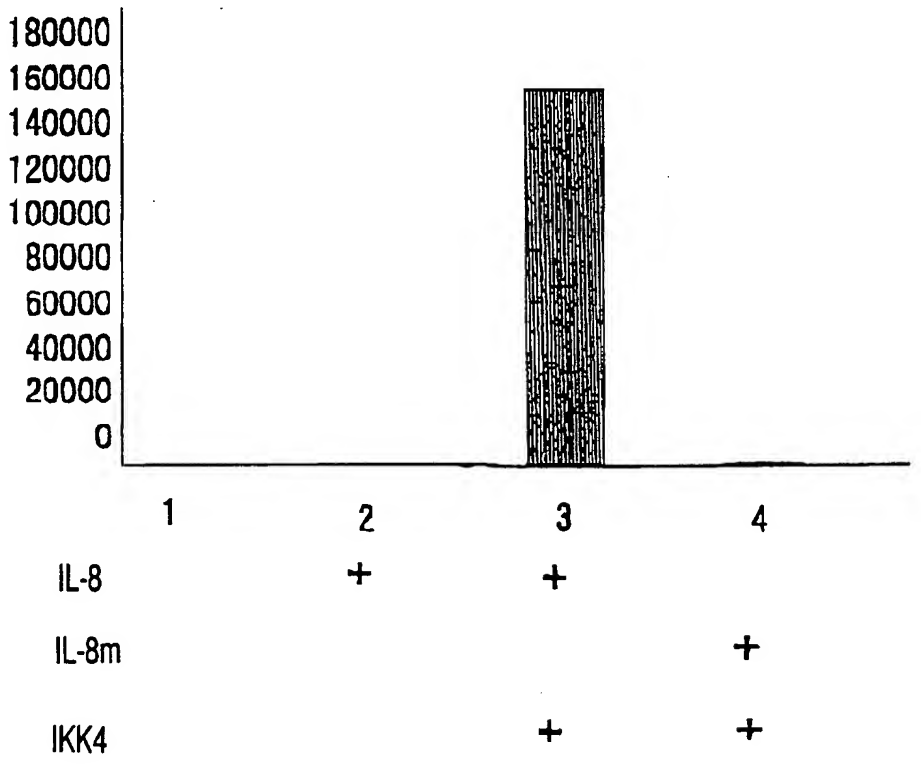

E.Coli (GST-IKK3 transformant)
Cell Lysates
Affinity Purification
GST
IKK3
Direct
phosphory lation
TRIP9
GST
GST
IKK3
ATP
ADP
P
P
TRIP9
GST
GST-IKK3
GST
[γ- 32P] ATP
(TRIP9=1 kappa B)
SDS-PAGE
Autoradiography
- GST-IKK3
- GST-TRIP9,GST-TRIP9 mutant
- GST
GST-IKK3 + + + +
GST +
GST-TRIP9 +
GST-TRIP9 mutant +
1 2 3 4 5 6
— GST-IKK4
— GST-TRIP
— GST
GST-IKK4 - - - + + +
GST + + + + - -
GST-TRIP9 - + - - + -
GST-TRIP9/AA - - + - - +

FIG. 6



IKK4 controls an essential step in the NF-kB signalling pathway. Hela cells were transiently transfected with IL-8 or IL-8 mutant luciferase reporter gene plasmid, and expression vector encoding double T7-tagged IKK4 (IKK4), or with a control vector. Luciferase activities were determined and normalized on the basis of □-galactosidase expression from cotransfected pact-□-Gal.

FIG. 7

```
IKK1    MERPPGLRPGAGGPWEMRERLGTGGFGNVCLYQHRELDLKIAIKSCRLELSTKNRERWCH  60
IKK2    MSWSPSLTTQTCGAWEMKERLGTGGFGNVIRWHNQETGEQIAIKQCRQELSPRNRERWCL  60
IKK3    -------MQSTANYLWHTDDLLGQGATASVYKARNKKSGELVAVKVFNTTSYLRPREVQVR  54
IKK4    ------MQSTSNHLWLLSDILGQGATANVFRGRHKKTGDLFAIKVFNNISFLRPVDVQMR  54

IKK1    EIQIMKKLNHANVVKACDVPEELN-ILIHDVPLLAMEYCSGGDLRKLLNKPENCCGLKES  119
IKK2    EIQIMRRLTHPNVVAARDVPEGMQNLAPNDLPLLAMEYCQGGDLRKYLNQFENCCGLREG  120
IKK3    EFEVLRKLNHQNIVKLFAVEETGG----SRQKVLVMEYCSSGSLLSVLESPENAFGLPED  110
IKK4    EFEVLKKLNHKNIVKLFAIEEETT----TRHKVLIMEFCPCGSLYTVLEEPSNAYGLPES  110

IKK1    QILSLLSDIGSGIRYLHENKIIHRDLKPENIVLQDVG-GKIIHKIIDLGYAKDVDQGSLC  178
IKK2    AILTLLSDIASALRYLHENRIIHRDLKPENIVLQQGE-QRLIHKIIDLGYAKELDQGSLC  179
IKK3    EFLVVLRCVVAGMNHLRENGIVHRDIKPGNIMRLVGEEGQSIYKLTDFGAARELDDDEKF  170
IKK4    EFLIVLRDVVGGMNHLRENGIVHRDIKPGNIMRVIGEDGQSVYKLTDFGAARELEDDEQF  170

IKK1    TSFVGTLQYLAPELFE--------NKPYTATVDYWSFGTMVFECIAGYRPFLHHLQP---  227
IKK2    TSFVGTLQYLAPELLE--------QQKYTVTVDYWSFGTLAFECITGFRPFLPNWQP---  228
IKK3    VSVYGTEEYLHPDMYERAVLRKPQQKAFGVTVDLWSIGVTLYHAATGSLPFTPFGGPRRN  230
IKK4    VSLYGTEEYLHPDMYERAVLRKDHQKKYGATVDLWSIGVTFYHAATGSLPFRPFEGPRRN  230

IKK1    --FTWHEKIKKKDPKCIFACEEMSGEVRFSSHLPQPNSLCSLIVEPMENWLQLMLNWDPQ  285
IKK2    --VQWHSKVRQKSEVDIVVSEDLNGTVKFSSSLPYPNNLNSVLAERLEKWLQLMLMWHPR  286
IKK3    KEIMYRITTEKPAGAIAGAQRRENGPLEWSYTLPITCQLSLGLQSQLVPILANILEVE--  288
IKK4    KEVMYKIITGKPSGAISGVQKAENGPIDWSGDMPVSGSLSRGLQVLLTPVLANILEAD--  288

IKK1    QRGGPVDLTLKQPRGFVLMDHILNLKIVHILNMTSAKIISFLLPPDESLHSLQSRIERET  345
IKK2    QRG--TDPTYGPNGGFKALDDILNLKLVHILNMVTGTIHTYPVTEDESLQSLKARIQQDT  344
IKK3    QAK-----CWGFDQFFAETSDILQRVVVHVFSLSQAVLHHIYIHAHNTIAIFQEAVHKQT  343
IKK4    QEK-----CWGFDQFFAETSDILHRMVIHVFSLQQMTAHKIYIHSYNTATIFHELVYKQT  343

IKK1    GINTGSQELLSETG-ISLDPRKPASQCVLDG----VRGCDSYMVYLFDKSKTVYEGPFAS  400
IKK2    GIPEEDQELLQEAG-LALIPDKPATQCISDGKLNEGHTLDMDLVFLFDNSKITYETQISP  403
IKK3    SVAPRHQEYLFEGHLCVLEPSV-SAQHIAHT--------TASSPLTLFSTA---IPKGLAF  392
IKK4    KIISSNQELIYEGRRLVLEPGR-LAQHFPKT---------TEENPIFVVSRE---PLNTIGL  392

IKK1    RSLSDCVNYIVQDSKIQLPIIQLRKVWAEAVHYVSGLKEDYSRLFQGQRAAMLSLLRYNA  460
IKK2    RPQPESVSCILQEPKRNLAFFQLRKVWGQVWHSIQTLKEDCNRLQQGQRAAMMNLLRNNS  463
IKK3    RDPALDVPKFVPKVDLQADYNTAKGVLGAGYQALRLARALLD----GQELMFRGLHWVME  448
IKK4    IYEKISLPKVHPRYDLDGDASMAKAITGVVCYACRIASTLLL----YQELMRKGIRWLIE  448
```

FIG. 8

```
IKK1    NLTKMKNTLISASQQLKAKLEFFHKSIQLDLERYSEQMTYGISSEKMLKAWKEMEEKAIH  520
IKK2    CLSKMKNSMASMSQQLKAKLDFFKTSIQIDLEKYSEQTEFGITSDKLLLAWREMEQAVEL  523
IKK3    VLQATCRRTLEVARTSLLYLSSSLGTERFSSVAGTPEIQELKAAAELRSRLRTLAEVLSR  508
IKK4    LIKDDYNETVHKKTEVVITLDFCIRNIEKTVKVYEKLMKINLEAAELG-EISDIHTKLLR  507

IKK1    YAEVGVIGYLEDQIMSLHAEIMEL---------QKSPYG-RRQGDLMESLEQRAIDLYKQ  570
IKK2    CGRENEVKLLVERMMALQTDIVDL---------QRSPMG-RKQGGTLDDLEEQARELYRR  573
IKK3    CSQN--ITETQESLSSLNRELVKS---------RDQVHE-DRSIQQIQCCLDKMNFIYKQ  556
IKK4    LSSS--QGTIETSLQDIDSRLSPGGSLADAWAHQEGTHPKDRNVEKLQVLLNCMTEIYYQ  565

IKK1    LKHRPSD-HSYSDSTEMVKIIVHTVQSQDRVLKELFGHLSKLLGCKQKIIDLLPKVEVAL  629
IKK2    LREKPRDQRTEGDSQEMVRLLLQAIQSFEKKVRVIYTQLSKTVVCKQKALELLPKVEEVV  633
IKK3    FKKSRMR-PGLGYNEEQ----IHKLDKVNFSHLAKRLLQVFQEECVQKYQASLVTHGKRM  611
IKK4    FKKDKAE-RRLAYNEEQ----IHKFDKQKLYYHATKAMTHFTDECVKKYEAFLNKSEEWI  620

IKK1    SNIKEADNTVMFMQGKRQKEIWHLLKIACTQSSARSLVGSSLEGAVTPQTSAWLPPTSAE  689
IKK2    SLMNEDEKTVVRLQEKRQKELWNLLKIACSK--VRGPVSGSPD---SMNASRLSQPGQLM  688
IKK3    RVVHETRNHLRLVGCSVAACNTEAQGVQESLSKLLEELSHQLLQDRAKGAQASPPPIAPY  671
IKK4    RKMLHLRKQLLSLTNQCFDIEEEVSKYQEYTNELQETLPQKMFTASS-GIKHTMTPIYPS  679

IKK1    HDHSLSCVVTPQDGETSAQMIEENLNCLGHLSTIIHEANEEQGNSMMNLDWSWLT-----  745
IKK2    SQPSTASNSLPEPAKKSEELVAEAHNLCTLLENAIQDTVREQDQSFTALDWSWLQTEEEE  748
IKK3    PSPTRKDLLLH-----MQELCEGMKLLASDLLDNNR--IIERLNRVPAPPDV--------  716
IKK4    -SNTLVEMTLG-----MKKLKEEMEGVVKELAENNH--ILERFGSLTMDGGLRNVDCL--  729

IKK1    --------
IKK2    HSCLEQAS    756
IKK3    --------
IKK4    --------
```

Predicted amino acid sequence of IKK4.

The potential kinase domain (KD) and helix-loop-helix (HLH) are boxed. The potential leucine zipper are underlined. Asterisks and dots indicate identical and similar amino acids, respectively. Numbers in the right indicate position of the amino acids.

FIG. 9

ikk4.seq Length: 3372

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  51 TTGGGCCCTC TAGATGCATG CTCGAGCGGC CGCCAGTGTG ATGGATATCT
 101 GCAGAATTCG CCCTTCCATC CTAATACGAC TCACTATAGG GCTCGAGCGG
 151 CCGCCCCGGGC AGGTCCAGGG CTTGCGAAGC CGGAAGTGTC CTGAGTCTCG
 201 AGGAGGCCGC GGGAGCCCGC CGGCGGTGGC GCGGCGGAGA CCCGGCTGGT
 251 ATAACAAGAG GATTGCCTGA TCCAGCCAAG ATGCAGAGCA CTTCTAATCA
 301 TCTGTGGCTT TTATCTGATA TTTTAGGCCA AGGAGCTACT GCAAATGTCT
 351 TTCGTGGAAG ACATAAGAAA ACTGGTGATT TATTTGCTAT CAAAGTATTT
 401 AATAACATAA GCTTCCTTCG TCCAGTGGAT GTTCAAATGA GAGAATTTGA
 451 AGTGTTGAAA AAACTCAATC ACAAAAATAT TGTCAAATTA TTTGCTATTG
 501 AAGAGGAGAC AACAACAAGA CATAAAGTAC TTATTATGGA ATTTTGTCCA
 551 TGTGGGAGTT TATACACTGT TTTAGAAGAA CCTTCTAATG CCTATGGACT
 601 ACCAGAATCT GAATTCTTAA TTGTTTTGCG AGATGTGGTG GGTGGAATGA
 651 ATCATCTACG AGAGAATGGT ATAGTGCACC GTGATATCAA GCCAGGAAAT
 701 ATCATGCGTG TTATAGGGGA AGATGGACAG TCTGTGTACA AACTCACAGA
 751 TTTTGGTGCA GCTAGAGAAT TAGAAGATGA TGAGCAGTTT GTTTCTCTGT
 801 ATGGCACAGA AGAATATTTG CACCCTGATA TGTATGAGAG AGCAGTCCTA
 851 AGAAAAGATC ATCAGAAGAA ATATGGAGCA ACAGTTGATC TTTGGAGCAT
 901 TGGGGTAACA TTTTACCATG CAGCTACTGG ATCACTGCCA TTTAGACCCT
 951 TTGAAGGGCC TCGTAGGAAT AAAGAAGTGA TGTATAAAAT AATTACAGGA
1001 AAGCCTTCTG GTGCAATATC TGGAGTACAG AAAGCAGAAA ATGGACCAAT
1051 TGACTGGAGT GGAGACATGC CTGTTTCTTG CAGTCTTTCT CGGGGTCTTC
```

FIG. 10

1101 AGGTTCTACT TACCCCTGTT CTTGCAAACA TCCTTGAAGC AGATCAGGAA

1151 AAGTGTTGGG GTTTTGACCA GTTTTTTGCA GAAACTAGTG ATATACTTCA

1201 CCGAATGGTA ATTCATGTTT TTTCGCTACA ACAAATGACA GCTCATAAGA

1251 TTTATATACA TAGCTATAAT ACTGCTACTA TATTTCATGA ACTGGTATAT

1301 AAACAAACCA AAATTATTTC TTCAAATCAA GAACTTATCT ACGAAGGGCG

1351 ACGCTTAGTC TTAGAACCTG GAAGGCTGGC ACAACATTTC CCTAAAACTA

1401 CTGAGGAAAA CCCTATATTT GTAGTAAGCC GGGAACCTCT GAATACCATA

1451 GGATTAATAT ATGAAAAAAT TTCCCTCCCT AAAGTACATC CACGTTATGA

1501 TTTAGACGGG GATGCTAGCA TGGCTAAGGC AATAACAGGG GTTGTGTGTT

1551 ATGCCTGCAG AATTGCCAGT ACCTTACTGC TTTATCAGGA ATTAATGCGA

1601 AAGGGGATAC GATGGCTGAT TGAATTAATT AAAGATGATT ACAATGAAAC

1651 TGTTCACAAA AAGACAGAAG TTGTGATCAC ATTGGATTTC TGTATCAGAA

1701 ACATTGAAAA AACTGTGAAA GTATATGAAA AGTTGATGAA GATCAACCTG

1751 GAAGCGGCAG AGTTAGGTGA AATTTCAGAC ATACACACCA AATTGTTGAG

1801 ACTTTCCAGT TCTCAGGGAA CAATAGAAAC CAGTCTTCAG GATATCGACA

1851 GCAGATTATC TCCAGGTGGA TCACTGGCAG ACGCATGGGC ACATCAAGAA

1901 GGCACTCATC CGAAAGACAG AAATGTAGAA AAACTACAAG TCCTGTTAAA

1951 TTGCATGACA GAGATTTACT ATCAGTTCAA AAAAGACAAA GCAGAACGTA

2001 GATTAGCTTA TAATGAAGAA CAAATCCACA AATTTGATAA GCAAAAACTG

2051 TATTACCATG CCACAAAAGC TATGACGCAC TTTACAGATG AATGTGTTAA

2101 AAAGTATGAG GCATTTTTGA ATAAGTCAGA ACAATGGATA AGAAAGATGC

2151 TTCATCTTAG GAAACAGTTA TTATCGCTCA CTAATCAGTG TTTTGATATT

2201 GAAGAAGAAG TATCAAAATA TCAAGAATAT ACTAATGAGT TACAAGAAAC

FIG. 11

2251 TCTGCCTCAG AAAATGTTTA CAGCTTCCAG TGGAATCAAA CATACCATGA

2301 CCCCAATTTA TCCAAGTTCT AACACATTAG TAGAAATGAC TCTTGGTATG

2351 AAGAAATTAA AGGAAGAGAT GGAAGGGGTG GTTAAAGAAC TTGCTGAAAA

2401 TAACCACATT TTAGAAAGGT TTGGCTCTTT AACCATGGAT GGTGGCCTTC

2451 GCAACGTTGA CTGTCTTTAG CTTTCTAATA GAAGTTTAAG AAAAGTTTCC

2501 GTTTGCACAA GAAAATAACG CTTGGGCATT AAATGAATGC CTTTATAGAT

2551 AGTCACTTGT TTCTACAATT CAGTATTTGA TGTGGTCGTG TAAATATGTA

2601 CAATATTGTA AATACATAAA AAATATACAA ATTTTTGGCT GCTGTGAAGA

2651 TGTAATTTTA TCTTTTAACA TTTATAATTA TATGAGGAAA TTTGACCTCA

2701 GTGATCACGA GAAGAAAGCC ATGACCGACC AATATGTTGA CATACTGATC

2751 CTCTACTCTG AGTGGGGCTA AATAAGTTAT TTTCTCTGAC CGCCTACTGG

2801 AAATATTTTT AAGTGGAACC AAAATAGGCA TCCTTACAAA TCAGGAAGAC

2851 TGACTTCACA CGTTTGTAAA TGGTAGAACG GTGGCTACTG TGAGTGGGGA

2901 GCAGAACCGC ACCACTGTTA TACTGGGATA ACAATTTTTT TGAGAAGGAT

2951 AAAGTGGCAT TATTTTATTT TACAAGGTGC CCAGATCCCA GTTATCCTTG

3001 TATCCATGTA ATTTCAGATG AATTATTAAG CAAACATTTT AAAGTGAATT

3051 CATTATTAAA AACTATTCAT TTTTTTCCTT TGGCCATAAA TGTGTAATTG

3101 TCATTAAAAT TCTAAGGTCA TTTCAACTGT TTTAAGCTGT ATTTCTTTAA

3151 TTCTGCTTAC TATTTCATGG AAAAAAATAA ATTTCTCAAT TTTAAAAAAA

3201 AAAAAAAAAA AAAAAAAAGC GTTCTAGAAT TCAGCGGCCG CTGAATTCTA

3251 GACCTGCCCG GGCGGCCGCT CGAGCCCTAT AGTGAGTCGT ATTAGGATGG

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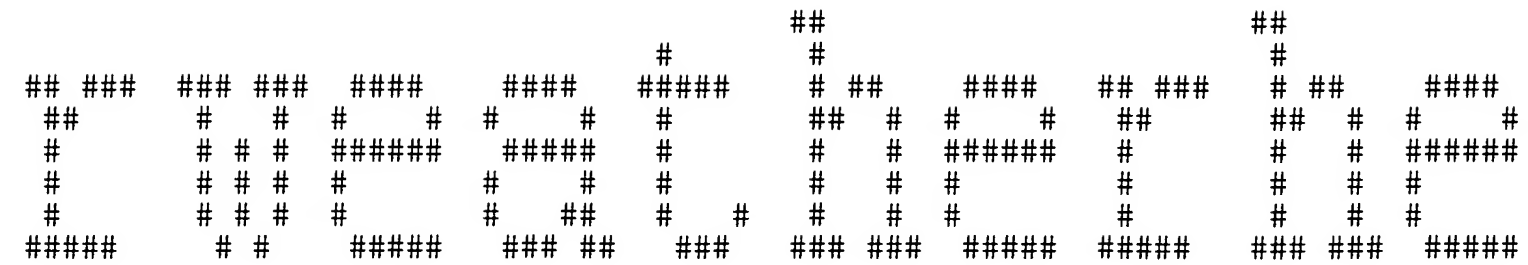
FIG. 12



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complementary to human ribonucleotide reductase R2
mRNA

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<210> 12
<211> 20
<212> DNA
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<220>
<223> AS-II-110-20 antisense oligonucleotides
complementary to human ribonucleotide reductase R2
mRNA

<400> 12
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<210> 13
<211> 20
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<220>
<223> AS-II-114-20 antisense oligonucleotides
complementary to human ribonucleotide reductase R2
mRNA

<400> 13
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<210> 14

<211> 12
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<220>
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complementary to human ribonucleotide reductase R2
mRNA

<400> 14
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<210> 15
<211> 20
<212> DNA
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<220>
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complementary to human ribonucleotide reductase R2
mRNA

<400> 15
tgggacaggg tgcacgggcg 20

<210> 16
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<223> AS-II-134-20 antisense oligonucleotides
complementary to human ribonucleotide reductase R2
mRNA

<400> 16
gacggctggg acagggtgca 20

<210> 17
<211> 20
<212> DNA
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<223> AS-II-151-20 antisense oligonucleotides
complementary to human ribonucleotide reductase R2
mRNA

<400> 17
gagcagccag gacaggacgg 20

<210> 18
<211> 20
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<220>
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complementary to human ribonucleotide reductase R2
mRNA

<400> 18
gcgaagcaga gcgagcagcc 20

<210> 19
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<220>
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complementary to human ribonucleotide reductase R2
mRNA

<400> 19
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<210> 20
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<220>
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complementary to human ribonucleotide reductase R2
mRNA

<400> 20
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<210> 21
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complementary to human ribonucleotide reductase R2
mRNA

<400> 21
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<210> 22
<211> 20
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complementary to human ribonucleotide reductase R2
mRNA

<400> 22
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<210> 23
<211> 20
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complementary to human ribonucleotide reductase R2
mRNA

<400> 23
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<210> 24
<211> 20
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complementary to human ribonucleotide reductase R2
mRNA

<400> 24
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<210> 25
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<220>
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complementary to human ribonucleotide reductase R2
mRNA

<400> 25
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<210> 26
<211> 20
<212> DNA
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<220>
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complementary to human ribonucleotide reductase R2
mRNA

<400> 26
cggcggcgtg ttctccttgt 20

<210> 27
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<220>
<223> AS-II-288-12 antisense oligonucleotides
complementary to human ribonucleotide reductase R2
mRNA

<400> 27
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<210> 28
<211> 20
<212> DNA
<213> Artificial Sequence

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complementary to human ribonucleotide reductase R2

mRNA

<400> 28
tcctcgcggt cttgctggcc 20

<210> 29
<211> 20
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complementary to human ribonucleotide reductase R2
mRNA

<400> 29
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<210> 30
<211> 20
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<213> Artificial Sequence

<220>
<223> AS-II-362-20 antisense oligonucleotides
complementary to human ribonucleotide reductase R2
mRNA

<400> 30
ctgctttagt tttcggctcc 20

<210> 31
<211> 17
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<220>
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complementary to human ribonucleotide reductase R2
mRNA

<400> 31
cggctcatcc tccacgc 17

<210> 32
<211> 20
<212> DNA
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<220>
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complementary to human ribonucleotide reductase R2
mRNA

<400> 32
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<210> 33
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> AS-II-412-20 antisense oligonucleotides
complementary to human ribonucleotide reductase R2
mRNA

<400> 33
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<210> 34
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<220>
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complementary to human ribonucleotide reductase R2
mRNA

<400> 34
aagcggcggg ggttttctct 20

<210> 35
<211> 20
<212> DNA
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<220>
<223> AS-II-425-20 antisense oligonucleotides
complementary to human ribonucleotide reductase R2
mRNA

<400> 35
ggaagatgac aaagcggcgg 20

<210> 36
<211> 20
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complementary to human ribonucleotide reductase R2
mRNA

<400> 36
atggtactcg atggggaaga 20

<210> 37
<211> 20
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<220>
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complementary to human ribonucleotide reductase R2
mRNA

<400> 37
agcctctgcc ttcttataca 20

<210> 38
<211> 20
<212> DNA
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<220>
<223> AS-II-494-20 antisense oligonucleotides
complementary to human ribonucleotide reductase R2
mRNA

<400> 38
cctcctcggc ggtccaaaag 20

<210> 39
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<220>
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complementary to human ribonucleotide reductase R2
mRNA

<400> 39
tcctcggcgg tccaaa 16

<210> 40
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<220>
<223> AS-II-549-20 antisense oligonucleotides
complementary to human ribonucleotide reductase R2
mRNA

<400> 40
tatctctcct cgggtttcag 20

<210> 41
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
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complementary to human ribonucleotide reductase R2
mRNA

<400> 41
gcaaagaaag ccagaacatg 20

<210> 42
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complementary to human ribonucleotide reductase R2
mRNA

<400> 42
tcgctccacc aagttttcat 20

<210> 43
<211> 20

<212> DNA
<213> Artificial Sequence

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<223> AS-II-634-20 antisense oligonucleotides
complementary to human ribonucleotide reductase R2
mRNA

<400> 43
aacttcttgg ctaaatcgct 20

<210> 44
<211> 20
<212> DNA
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<223> AS-II-667-20 antisense oligonucleotides
complementary to human ribonucleotide reductase R2
mRNA

<400> 44
gaagccatag aaacagcggg 20

<210> 45
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complementary to human ribonucleotide reductase R2
mRNA

<400> 45
gacacaaggc atcgtttcaa 20

<210> 46
<211> 20
<212> DNA
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<220>
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complementary to human ribonucleotide reductase R2
mRNA

<400> 46
tctgccttct tcttgacaca 20

<210> 47
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<212> DNA
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mRNA

<400> 47
atccagcgca aggcccagtc 20

<210> 48
<211> 20
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<220>
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complementary to human ribonucleotide reductase R2
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<400> 48
gcaaaggcta caacacgttc 20

<210> 49
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complementary to human ribonucleotide reductase R2
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<400> 49
aaccggaaaa gaaaatgcct 20

<210> 50
<211> 20
<212> DNA
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<220>
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complementary to human ribonucleotide reductase R2
mRNA

<400> 50
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<210> 51
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complementary to human ribonucleotide reductase R2
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<400> 51
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<210> 52
<211> 20
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complementary to human ribonucleotide reductase R2
mRNA

<400> 52

tgtaaaccct catctctgct 20

<210> 53
<211> 20
<212> DNA
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<220>
<223> AS-II-1001-20 antisense oligonucleotides
complementary to human ribonucleotide reductase R2
mRNA

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<210> 54
<211> 20
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<220>
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complementary to human ribonucleotide reductase R2
mRNA

<400> 54
gaacatcagg caagcaaaat 20

<210> 55
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complementary to human ribonucleotide reductase R2
mRNA

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<210> 56
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ctctctcctc cgatggtttg 20

<210> 57
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mRNA

<400> 57
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<210> 58
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complementary to human ribonucleotide reductase R2
mRNA

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<210> 59
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<220>
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complementary to human ribonucleotide reductase R2
mRNA

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cccagttcca gcataagtct 20

<210> 60
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complementary to human ribonucleotide reductase R2
mRNA

<400> 60
aaaaccttgc taaaacccag 20

<210> 61
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<220>
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complementary to human ribonucleotide reductase R2
mRNA

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<210> 62
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complementary to human ribonucleotide reductase R2 mRNA

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<210> 63
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<210> 64
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<220>
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<210> 65
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<400> 65
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<210> 66
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<220>
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<400> 66
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<210> 67
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<220>
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complementary to human ribonucleotide reductase R2
mRNA

<400> 67
gcatccaagg taaaagaatt 20

<210> 68
<211> 20
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<220>
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complementary to human ribonucleotide reductase R2
mRNA

<400> 68
tcagcatcca aggtaaaaga 20

<210> 69
<211> 20
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complementary to human ribonucleotide reductase R2
mRNA

<400> 69
gaagtcagca tccaaggtaa 20

<210> 70
<211> 20
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complementary to human ribonucleotide reductase R2
mRNA

<400> 70
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<210> 71
<211> 20
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complementary to human ribonucleotide reductase R2
mRNA

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gcacatcttc agttcattta 20

<210> 72
<211> 20
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<213> Artificial Sequence

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complementary to human ribonucleotide reductase R2
mRNA

<400> 72
gggcacatct tcagttcatt 20

<210> 73
<211> 20
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<220>
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complementary to human ribonucleotide reductase R2
mRNA

<400> 73
aaaaatcagc caagtaaggg 20

<210> 74
<211> 20
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complementary to human ribonucleotide reductase R2
mRNA

<400> 74
atggaaaaaa aaaatcagcc 20

<210> 75
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<220>
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complementary to human ribonucleotide reductase R2
mRNA

<400> 75
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<210> 76
<211> 20
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<220>
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complementary to human ribonucleotide reductase R2
mRNA

<400> 76
aggactggtt gtgaggtagc 20

<210> 77

<211> 20
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<220>
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complementary to human ribonucleotide reductase R2
mRNA

<400> 77
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<210> 78
<211> 20
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<220>
<223> AS-II-1538-20 antisense oligonucleotides
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mRNA

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ttctggcaaa aggtgatact 20

<210> 79
<211> 20
<212> DNA
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<223> AS-II-1560-20 antisense oligonucleotides
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mRNA

<400> 79
gtaagtcaca gccagccagg 20

<210> 80
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<220>
<223> AS-II-1581-20 antisense oligonucleotides
complementary to human ribonucleotide reductase R2
mRNA

<400> 80
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<210> 81
<211> 20
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<220>
<223> AS-II-1659-20 antisense oligonucleotides
complementary to human ribonucleotide reductase R2
mRNA

<400> 81
tggctgtgct ggttaaagga 20

<210> 82
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complementary to human ribonucleotide reductase R2
mRNA

<400> 82
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<210> 83
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mRNA

<400> 83
attaaaatct gcgttgaagc 20

<210> 84
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complementary to human ribonucleotide reductase R2
mRNA

<400> 84
tatcgccgcc gtgagtacaa 20

<210> 85
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complementary to human ribonucleotide reductase R2
mRNA

<400> 85
gctattatcg ccgccgtgag 20

<210> 86
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<220>
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complementary to human ribonucleotide reductase R2
mRNA

<400> 86
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<210> 87
<211> 20
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mRNA

<400> 87
gaaaccaaat aaatcaagct 20

<210> 88
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mRNA

<400> 88
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<210> 89
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mRNA

<400> 89
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<210> 90
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mRNA

<400> 90
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<210> 91
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mRNA

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<210> 92
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mRNA

<400> 92
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<210> 93
<211> 20
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<223> AS-II-2044-20 antisense oligonucleotides
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mRNA

<400> 93
tcagaggggg cagagaatcc 20

<210> 94
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<220>
<223> AS-II-2067-20 antisense oligonucleotides
complementary to human ribonucleotide reductase R2
mRNA

<400> 94
tcctttatcc cacaacactc 20

<210> 95
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> AS-II-2083-20 antisense oligonucleotides
complementary to human ribonucleotide reductase R2
mRNA

<400> 95
ccttgccctg agagattcct 20

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<223> Partially Phosphorothioated AS-II-2083-20 antisense oligonucleotides
complementary to human ribonucleotide reductase R2
mRNA

<400> 96
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<210> 97
<211> 20
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<220>
<223> AS-II-2128-20 antisense oligonucleotides
complementary to human ribonucleotide reductase R2
mRNA

<400> 97
ggcccagatc acccctaaat 20

<210> 98
<211> 20
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<220>
<223> AS-II-2151-20 antisense oligonucleotides
complementary to human ribonucleotide reductase R2
mRNA

<400> 98
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<210> 99
<211> 20
<212> DNA
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<220>
<223> AS-II-2164-20 antisense oligonucleotides
complementary to human ribonucleotide reductase R2
mRNA

<400> 99
gagaaataaa atgaaacggc 20

<210> 100
<211> 20
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complementary to human ribonucleotide reductase R2
mRNA

<400> 100
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<210> 101
<211> 20
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<220>
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complementary to human ribonucleotide reductase R2
mRNA

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gctcccacat atgaaaactc 20

<210> 102
<211> 20
<212> DNA
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<220>
<223> AS-II-2372-20 antisense oligonucleotides
complementary to human ribonucleotide reductase R2
mRNA

<400> 102
cacacaacct acttacacca 20

<210> 103
<211> 20
<212> DNA
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<220>
<223> Antisense oligonucleotides complementary to human
ribonucleotide reductase R2 mRNA

<400> 103
tcctggaaga tcctcctcgc 20

<210> 104
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<213> Artificial Sequence

<220>
<223> Antisense oligonucleotides complementary to human
ribonucleotide reductase R2 mRNA

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gtgaggggtc gcccgtgcac cctgtcccag ccgtcctgtc ctggctgctc gctctgcttc 180
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agctgcagct ctcgccgctg aaggggctca gcttggtcga caaggagaac acgccgccgg 300
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